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**WO 01/97856 A2**

(54) Title: **AN ORPHAN NUCLEAR RECEPTOR**

(57) Abstract: The present invention relates to a novel human orphan nuclear receptor that binds to a cytochrome P-450 monooxygenase (CYP) promoter and that is activated by compounds that induce CYP gene expression. The invention further relates to nucleic acid sequences encoding such a receptor, to methods of making the receptor and to methods of using the receptor and nucleic acid sequences encoding same. The invention also relates to non-human animals transformed to express the human receptor and to methods of using such animals to screen compounds for drug interactions and toxicities.

## AN ORPHAN NUCLEAR RECEPTOR

The present invention is a continuation-in-part of Application No. 09/276,935, filed March 26, 1999, the entire disclosure of that application being  
5 incorporated herein by reference. Application No. 09/276,935 claims priority from Provisional Application No. 60/079,593, filed March 27, 1998, the entire contents of that provisional application also being incorporated herein by reference.

10

### TECHNICAL FIELD

The present invention relates to a novel human orphan nuclear receptor that binds to a cytochrome P-450 monooxygenase (CYP) promoter and that is activated by compounds that induce CYP gene  
15 expression. The invention further relates to nucleic acid sequences encoding such a receptor, to methods of making the receptor and to methods of using the receptor and nucleic acid sequences encoding same. The invention also relates to non-  
20 human animals transformed to express the human receptor and to methods of using such animals to screen compounds for drug interactions and toxicities.

### BACKGROUND OF THE INVENTION

25 Members of the cytochrome P-450 (CYP) family of hemoproteins are critical in the oxidative metabolism of a wide variety of endogenous

substances and xenobiotics, including various carcinogens and toxins (Nebert et al, Ann. Rev. Biochem. 56:945-993 (1987)). In man, the CYP3A4 monooxygenase plays a major role in the  
5 biotransformation of drugs due to its abundance in liver and intestine and its broad substrate specificity. CYP3A4 catalyzes the metabolism of >60% of all drugs that are in use including  
steroids, immunosuppressive agents, imidazole  
10 antimycotics, and macrolide antibiotics (Maurel, P. in Cytochromes P450: metabolic and toxicological aspects (ed. Ioannides, C.) 241-270 (CRC Press, Inc., Boca Raton, FL, 1996)).

Expression of the CYP3A4 gene is markedly  
15 induced both *in vivo* and in primary hepatocytes in response to treatment with a variety of compounds. Many of the most efficacious inducers of CYP3A4 expression are commonly used drugs such as the glucocorticoid dexamethasone, the antibiotic  
20 rifampicin, the antimycotic clotrimazole, and the hypocholesterolemic agent lovastatin (Maurel, P. in Cytochromes P450: metabolic and toxicological aspects (ed. Ioannides, C.) 241-270 (CRC Press, Inc., Boca Raton, FL, 1996), Guzelian, P.S. in  
25 Microsomes and Drug Oxidations (eds. Miners, J.O., Birkett, D.J., Drew, R. & McManus, M.) 148-155 (Taylor and Francis, London, 1988)). The inducibility of CYP3A4 expression levels coupled with the broad substrate specificity of the CYP3A4  
30 protein represent the basis for many drug interactions in patients undergoing combination drug

therapy. While attempts have been made to develop in vivo and in vitro assays with which to profile the effects of compounds on CYP3A expression levels, these efforts have been hampered by species-specific effects that have limited the utility of using animals and their tissues for testing purposes. Thus, analysis of the effects of new compounds on CYP3A4 gene expression has been largely restricted to laborious assays involving human liver tissue.

10 Recently, efforts have been directed at understanding the molecular basis for the induction of CYP3A4 gene expression. The CYP3A4 promoter has been cloned and a 20 bp region residing approximately 150 bp upstream of the transcription  
15 initiation site shown to confer responsiveness to dexamethasone and rifampicin (Hashimoto et al, Eur. J. Biochem. 218:585-595 (1993), Barwick et al, Molec. Pharmacol. 50:10-16 (1996)). This region contains two copies of the AG(G/T)TCA motif  
20 recognized by members of the nuclear receptor superfamily, suggesting that a nuclear receptor might be responsible for mediating at least some of the effects of the chemical inducers of CYP3A4 expression. However, prior to the present  
25 invention, proteins that bind to this response element had not been characterized.

The present invention is based on the identification of a novel orphan nuclear receptor that binds to a response element in the CYP3A4  
30 promoter and that is activated by a range of compounds known to induce CYP3A4 expression. The

identification of this receptor makes possible assays that can be used to establish whether drugs will interact *in vivo*.

#### SUMMARY OF THE INVENTION

5       The present invention relates to a novel human orphan nuclear receptor, designated the human pregnane X receptor (hPXR), that binds to a CYP promoter, for example, the rifampicin/dexamethasone response element in the cytochrome P-450  
10 monooxygenase 3A4 (CYP3A4) promoter. The receptor is activated to modulate transcription of a CYP (e.g., CYP3A4) gene. The present invention further relates to nucleic acids encoding hPXR, including expression vectors that can be used to effect  
15 expression of the receptor in host cells. The invention also relates to host cells transformed with such expression vectors and to methods of using the receptor and receptor encoding sequences in assays designed to screen compounds (e.g., drugs)  
20 for their ability to modulate CYP (e.g., CYP3A4) gene expression. The invention also relates to non-human animals transformed to express the human receptor and to methods of using same in drug screens.

#### 25       BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1D. Molecular cloning of hPXR.  
(Fig. 1 A) Nucleotide (SEQ ID NO:13) and predicted amino acid (SEQ ID NO:14) sequences of hPXR.

(Fig. 1B) Amino acid sequence comparison between hPXR, mPXR1, Xenopus orphan nuclear receptor 1 (xONR1) (Smith et al, Nucl. Acids Res. 22:66-71 (1994)), and the human vitamin D receptor (hVDR).

5 Numbers indicate percent amino acid identity in the DBDs and LBDs. (Fig. 1C) The hPXR clone encodes a functional nuclear receptor. Transfection assays were performed with a pSG5-hPXR expression vector containing the wild-type 5' region of the hPXR cDNA

10 and a reporter plasmid containing four copies of the CYP3A1 DR3 PXRE. Cells were treated with vehicle alone (0.1% DMSO) or 10  $\mu$ M of dexamethasone-t-butylacetate. Cell extracts were subsequently assayed for CAT activity. Data points represent the

15 mean of assays performed in duplicate. (Fig. 1D) Translation of the full-length hPXR initiates at a non-AUG codon. *In vitro* transcription and translation were performed with the pSG5-hPXR expression vector containing the wild-type 5' region

20 of the hPXR cDNA or pSG5-hPXR AUG, in which the CUG codon at nucleotide positions 304-306 was modified to AUG. The 50 kD product synthesized when either template was used is indicated by the open arrow and the asterisk. Two shorter products which are likely

25 to represent translation initiation at methionine-56 and methionine-69 within the DBD are indicated by closed arrows. A longer translation product present at low levels is indicated by the bent arrow. Size markers (in kD) are indicated at left.

Figure 2. Northern blot analysis of hPXR expression pattern in adult tissues (left to right, heart (1), brain (2), placenta (3), lung (4), liver (5), skeletal muscle (6), kidney (7), pancreas (8), spleen (9), thymus (10), prostate (11), testis (12), ovary (13), small intestine (14), colon (15), PBL (16). RNA size markers (in kb) are indicated at left.

Figures 3A-3C. hPXR activates transcription through an IR6 element in the CYP3A4 promoter. (Fig. 3A) CV-1 cells were cotransfected with the (IR6)<sub>3</sub>-tk-CAT reporter plasmid in either the absence (-) or presence (+) of the pSG5-hPXR ATG expression plasmid and treated with vehicle alone (open bars) or 10  $\mu$ M dexamethasone-t-butylacetate (closed bars). Cell extracts were subsequently assayed for CAT activity. Data represent the mean of assays performed in triplicate  $\pm$  S.E. (Fig. 3B) Oligonucleotides used in band shift assays. The positions of nuclear receptor half-site motifs and mutations are indicated. (Fig. 3C) Band shift assays were performed with a radiolabeled oligonucleotide containing the CYP3A4 IR6 PXRE and hRXR and either hPXR (top panel) or mPXR1 (bottom panel). Unlabeled competitor oligonucleotides were added at a 10-fold or 50-fold molar excess as indicated.

Figures 4A-4C. hPXR is activated by structurally-distinct inducers of CYP3A4 gene

expression. (Fig. 4A) CV-1 cells were transfected with the pSG5-hPXR ATG-or pSG5-mPXR1 expression plasmids and the (IR6)<sub>3</sub>-tk-CAT reporter (left and middle panels, respectively), or the RS-hGR  
5 expression plasmid (Giguere et al, Cell 46:645-652 (1986)) and a reporter containing two copies of a consensus glucocorticoid response element upstream of tk-CAT (right panel). Cells were treated with  
1  $\mu$ M mevastatin or lovastatin, 100  $\mu$ M phenobarbital,  
10 or 10  $\mu$ M of the other compounds. Cell extracts were subsequently assayed for CAT activity. Data represent the mean of assays performed in triplicate  $\pm$  S.E. (Fig. 4B) Structures of representative compounds that activate hPXR. (Fig. 4C) CARLA was  
15 performed with bacterially-expressed GST-hPXR or GST-mPXR1 and [<sup>35</sup>S]SRC1.14 synthesized in vitro. [<sup>35</sup>S]SRC1.14 was mixed with either GST-hPXR or GST-mPXR1 in the presence of vehicle alone (1) (1% DMSO) or 10  $\mu$ M of dexamethasone-t-butylacetate (2),  
20 rifampicin (3), or clotrimazole (4). [<sup>35</sup>S]SRC1.14 complexed with GST-hPXR (top panel) or GST-mPXR1 (bottom panel) was precipitated with glutathione-sepharose beads.

Figure 5. Reaction scheme for production of  
25 [<sup>3</sup>H]GW-485801.

Figure 6. Plot of specific binding vs. concentration of [<sup>3</sup>H]GW-485801. K<sub>d</sub> = 370 nM.



DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a novel human nuclear receptor, hPXR. The invention further relates to nucleic acid sequences encoding hPXR, to  
5 constructs comprising such sequences, to host cells containing the constructs and to a method of producing hPXR using such host cells. The invention also relates to non-human animals transformed to express hPXR. The invention further relates to *in*  
10 *vivo* and *in vitro* assays that can be used to identify compounds that induce CYP expression. While the disclosure that follows makes specific reference to CYP3A4, it should be appreciated that the details (e.g., methods) provided find  
15 application in connection with other CYP genes as well.

hPXR is characterized as a protein comprising about 434 amino acids and having a molecular weight of about 49.7 kilodaltons. hPXR binds to a DNA  
20 response element in the CYP3A4 promoter as a heterodimer with the 9-cis retinoic acid receptor, RXR. hPXR is activated by compounds known to modulate CYP3A4 expression. The receptor is most abundantly expressed in liver but is also present in  
25 colon and small intestine.

One embodiment of the receptor of the invention has the amino acid sequence set forth in Figure 1, or an analog thereof (wherein the term analog is intended to indicate a naturally occurring human  
30 variant of the Figure 1 sequence), or a fragment

thereof, including fragments having at least one functional characteristic of hPXR (e.g., ligand binding or DNA binding). Preferred fragments include portions of the Figure 1 sequence at least  
5 30 consecutive amino acids in length, more preferably, at least 50 consecutive amino acids in length, and most preferably, at least 75 consecutive amino acids in length. Specific fragments include the ligand binding domain (that is, amino acids 141  
10 to 434 of the Figure 1 sequence) and the DNA binding domain (that is, amino acids 41 to 107 of the Figure 1 sequence) as well as the domain that is used for the ligand binding assay described in the Examples that follow (that is, amino acids 130-434 of the  
15 Figure 1 sequence). The invention also includes a protein comprising a domain sharing at least 80% amino acid sequence identity with the ligand binding domain of the Figure 1 sequence, more preferably, at least 85% amino acid sequence identity and, most  
20 preferably, at least 90% or 95%, 96%, 97%, 98% or 99% amino acid sequence identity with the ligand binding domain of the Figure 1 sequence (% sequence identity being determined, for example, by Basic Blast (version 2.0) available through the NCBI  
25 website <http://www.ncbi.nlm.nih.gov/>), and, advantageously, retaining the function of the Figure 1 sequence.

The receptor of the invention, or fragment thereof, can bear a detectable label (e.g., a  
30 radioactive or fluorescent label). The receptor, or receptor fragment, can also be bound to a solid

support, e.g., a glass or plastic particle, a plate, or a filter.

Nucleic acid sequences of the invention include DNA and RNA sequences encoding hPXR, for example, 5 hPXR having the amino acid sequence given in Figure 1, as well as nucleic acid sequences encoding analogs and fragments, of the Figure 1 amino acid sequence as defined above, and nucleic acid sequences encoding proteins comprising a domain 10 sharing at least 80% amino acid sequence identity (more preferably, at least 85%, or at least 90%, or at least 95%, or at least 96%, or at least 97%, or at least 98% or at least 99%) with the ligand binding domain of the Figure 1 sequence, as 15 described above. A specific nucleic acid sequence of the invention is that shown in Figure 1.

The hPXR encoding sequence can be present in a construct, for example, in an expression construct, operably linked to a promoter (e.g., the CMV, SV40, 20 Taq, T7 or LacO promoter). Such expression constructs are operative in a cell in culture (e.g., yeast, bacteria, insect or mammalian), to express the encoded hPXR, or fragment thereof. Preferred expression vectors include pGEX, pET, pFASTbacHT and 25 pSG5.

The invention also relates to cells in culture (e.g., yeast, bacteria or mammalian (for example, CV-1, HuH7, HepG2, or CaCo2 cells)) that are transformed with an above-described construct. 30 Transformation can be effected using any of a variety of standard techniques. Such cells can be

used in a method of making hPXR (or fragment thereof) by culturing same under conditions suitable for expression of the polypeptide product.

The invention further relates to chimeric  
5 receptors (or fusion proteins having a receptor component) (and encoding sequences) comprising at least a DNA-binding domain or a ligand-binding domain of hPXR, and a non-hPXR derived sequence. Non-hPXR derived sequences can be selected so as to  
10 be suitable for the purpose to be served by the chimeric receptor. Examples of such sequences include glutathione-S-transferase and the DNA binding domain of yeast transcription factor GAL4 and other DNA binding domains, e.g., DNA binding  
15 domains for the estrogen and glucocorticoid receptors. The chimeric receptor can bear a detectable label (e.g., a radioactive or fluorescent label). The chimeric receptor can also be bound to a solid support, e.g., a glass or plastic particle,  
20 a plate or a filter.

A further aspect of the invention relates to *in vitro* (cell-free) and *in vivo* (cell-based) assays that can be used to profile the effects of compounds (e.g., potential new drugs) on CYP3A4 levels. The  
25 inducibility of CYP3A4 levels, coupled with the broad substrate specificity of the CYP3A4 enzyme, represent the basis for many drug-drug interactions in patients undergoing multiple drug therapy. Ideally, new drugs would have little or no effect on  
30 CYP3A4 expression levels.

The assays of the invention can take any of a variety of forms. As compounds that activate hPXR function as inducers of CYP3A4 gene expression, hPXR binding and activation assays provide efficient means to identify compounds that can be expected to activate CYP3A4.

Binding assays of the invention include cell free assays in which hPXR, or the ligand binding domain thereof (alone or present as a fusion protein), is incubated with a test compound which, advantageously, bears a detectable label (e.g., a radioactive or fluorescent label). The hPXR, or ligand binding domain thereof, free or bound to test compound, is then separated from free test compound using any of a variety of techniques (e.g., using gel filtration chromatography (for example, on Sephadex G50 spin columns) or through capture on a hydroxyapatite resin). The amount of test compound bound to hPXR or ligand binding domain thereof, is then determined (for example, by liquid scintillation counting in the case of radiolabelled test compounds).

An alternative approach for detecting radiolabeled test compound bound to hPXR, or ligand binding domain thereof, is a scintillation proximity assay (SPA). In this assay, a bead (or other particle) is impregnated with scintillant and coated with a molecule that can capture the hPXR, or ligand binding domain thereof (e.g., streptavidin-coated beads can be used to capture biotinylated hPXR ligand binding domain). Radioactive counts are

detected only when the complex of radiolabeled test compound and the hPXR, or ligand binding domain thereof, is captured on the surface of the SPA bead, bringing the radioactive label into sufficient  
5 proximity to the scintillant to emit a signal. This approach has the advantage of not requiring the separation of free test compound from bound (Nichols et al, Anal. Biochem. 257:112-119 (1998)).

Assays to determine whether a test compound  
10 interacts with the hPXR ligand binding domain can also be performed via a competition binding assay. In this assay, hPXR, or ligand binding domain thereof, is incubated with a compound known to interact with hPXR, which compound, advantageously,  
15 bears a detectable label (e.g., a radioactive or fluorescent label (see Example 5 - Crabtree catalysts suitable for use in the synthetic approach described in Example 5 include those reported by Chen et al, J. Labelled Compd. Radiopharm. 39:291  
20 (1997) and Crabtree et al, Inorg. Synth. 28:56 (1990))). A test compound is added to the reaction and assayed for its ability to compete with the labeled compound for binding to hPXR, or ligand binding domain thereof. A standard assay format  
25 employing a step to separate free known (labeled) compound from bound, or an SPA format, can be used to assess the ability of the test compound to compete.

A further example of a binding assay in  
30 accordance with the invention is based on the finding that hPXR ligands induce the interaction of

hPXR ligand binding domain with coactivators (e.g., SRC1, TIF-1, TIF-2 or ACTR, or fragment thereof). To determine if a test compound activates hPXR, and thus induces CYP3A4 expression, the ligand binding domain of hPXR is prepared (e.g., expressed) as a fusion protein (e.g., with glutathione-S-transferase (GST), a histidine tag or a maltose binding protein). The fusion protein and coactivator (either or both advantageously labeled with a detectable label, e.g., a radiolabel or fluorescent tag) are incubated in the presence and absence of the test compound and the extent of binding of the coactivator to the fusion protein determined. The induction of interaction in the presence of the test compound is indicative of an hPXR activator.

hPXR activation assays in accordance with the invention can be carried out using full length hPXR and a reporter system comprising one or more copies of the DNA binding site recognized by the hPXR binding domain (see Example 3). Advantageously, however, the activation assays are conducted using established chimeric receptor systems. For example, the ligand binding domain of hPXR can be fused to the DNA binding domain of, for example, yeast transcription factor GAL4, or that of the estrogen or glucocorticoid receptor. An expression vector for the chimera (e.g., the GAL4-hPXR chimera) can be transfected into host cells (e.g., CV-1, HuH7, HepG2 or CaCo2 cells) together with a reporter construct. The reporter construct can comprise one or more (e.g., 5) copies of the DNA binding site recognized

by the binding domain present in the chimera (e.g., the GAL4 DNA binding site) driving expression of a reporter gene (e.g., CAT, SPAP or luciferase). Cells containing the constructs are then treated  
5 with either vehicle alone or vehicle containing test compound, and the level of expression of the reporter gene determined. In accordance with this assay, enhancement of expression of the reporter gene in the presence of the test compound indicates  
10 that the test compound activates hPXR and thus can function as an inducer of CYP3A4 gene expression. (See Example 4.)

Another format suitable for use in connection with the present invention is the yeast two-hybrid  
15 assay. This is an established approach to detect protein-protein interactions that is performed in yeast. Protein #1, representing the bait, is expressed in yeast as a chimera with a DNA binding domain (e.g., GAL4). Protein #2, representing the  
20 predator, is expressed in the same yeast cell as a chimera with a strong transcriptional activation domain. The interaction of bait and predator results in the activation of a reporter gene (e.g., luciferase or  $\beta$ -galactosidase) or the regulation of  
25 a selectable marker (e.g., LEU2 gene). This approach can be used as a screen to detect, for example, ligand-dependent interactions between hPXR1 and other proteins such as coactivator proteins (e.g., SRC1, TIF1, TIF2, ACTR) or fragments thereof.  
30 (Fields et al, Nature 340:245-246 (1989)).



Still another format is the ligand-induced complex formation (LIC) assay. This is an approach to detect ligand-mediated effects on nuclear receptor-DNA interactions. hPXR (or, minimally, the DNA and ligand binding domains thereof) can be incubated with its heterodimeric partner RXR in the presence of DNA representing an established hPXR/RXR binding site. Test compounds can be assayed for their ability to either enhance or interfere with binding of the hPXR/RXR heterodimer to DNA (Forman et al, Proc. Natl. Acad. Sci. USA 94:4312-4317 (1997)).

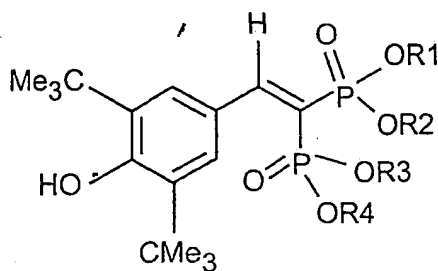
Compounds that bind PXR with a suitable  $pK_i$ , for example with a  $pK_i > 5$ , can be screened for selectivity for PXR versus other nuclear receptors (e.g., RXR) using standard binding assays. A compound that binds selectively to PXR (that is, has at least a 10 fold greater affinity for PXR, preferably, at least a 100 fold greater affinity for PXR, than, for example, the glucocorticoid receptor) and thereby affects the functional activity of PXR in a cell (e.g., a cell in culture, a cell present in a tissue or a cell present in a whole animal) can be used to associate PXR activity with a mammalian disease state. For example, a compound that activates PXR induces CYP3A. Thus, diseases in which CYP3A activity is important are associated with PXR, and compounds that activate or deactivate PXR may be useful in prevention or treatment of such diseases. By using the associating methods of this invention, new PXR-associated diseases can be

discovered. Once these new associations are discovered, new drugs for these diseases can be identified by screening for compounds that activate or deactivate PXR.

5 An example of a compound suitable for use in making disease associations in accordance with the method described above is the compound of formula I:

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wherein each of R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub> and R<sub>4</sub> is, independently, C<sub>1</sub>-C<sub>6</sub>alkyl (linear or branched), preferably, C<sub>2</sub> or C<sub>3</sub>alkyl (e.g., ethyl, n-propyl or  
30 iso-propyl), more preferably, C<sub>2</sub>alkyl. The compounds

can be labelled with a detectable label, e.g., a radiolabel, e.g., tritium.

A specific aspect of the invention relates to the identification of compounds that induce  
5 expression of CYP3A and that are useful in the treatment of cholestatic liver disease, including primary biliary cirrhosis (PBC), primary sclerosing cholangitis, autoimmune hepatitis with cholestatic features, autoimmune cholangitis, TPN-associated  
10 cholestatis, cholestasis of pregnancy, pediatric cholestatic syndromes, and drug-induced cholestasis (see Hofmann, Arch. Intern. Med. 159:2647 (1999)) also known as cholestasis. PBC affects 0.1% of women over the age of 40. The early stages of the  
15 disease are asymptomatic, but 80% of patients progress to the later stages of the disease which are characterized by lethargy and pruritis (itching). Eventually, patients become jaundiced and hepatic failure occurs. At this stage, the only  
20 treatment option is a liver transplant (Bar-Dayana et al, Immunol. Res. 18:117-123 (1998)). The only approved therapy for the early stages of PBC is the bile acid ursodeoxycholic acid (UDCA) which alleviates some of the symptoms of the disease,  
25 however, the effect of UDCA on disease progression is unknown (Angulo et al, Hepatology 29:1007-1012 (1999)). The mechanism of action of UDCA is unknown is unknown. Two observations indicate that PXR activators are useful in the treatment of PBC.  
30 First, the drug pregnenolone 16 $\alpha$  carbonitrile (PCN),

a known PXR activator, prevents chemically-induced cholestasis in rats (Selye, Proc. Soc. Exp. Biol. Med. 141:555-558 (1972)). Second, UDCA has been reported to induce expression of CYP3A in mice  
5 (Paolini et al, Hepatology 30:730-739 (1999)). As indicated above, assays described herein can be used to identify additional CYP3A inducers that can be used in the treatment of PBC. Compounds that induce CYP3A can be formulated using standard procedures  
10 and optimum administration regimens can be readily determined by one skilled in the art.

Another aspect of the invention relates to transgenic animals that express hPXR. For example, transgenic mice can be generated that express the  
15 hPXR gene as well as the endogenous mouse PXR gene. Mice can also be generated in which the endogenous PXR gene is knocked out and then replaced by the hPXR gene. Transgenic animals can be generated that express isoforms of hPXR as well as mutant alleles  
20 of the gene. Transgenic animals developed by these methods can be used to screen compounds for drug interactions and toxicities, and to study the regulation of CYP3A in vivo.

A further aspect of the present invention  
25 relates to diagnostic assays that can be used to screen for mutations in hPXR that alter the ability of the receptor to induce CYP3A4 gene expression. These assays can be based on the sequencing of the hPXR gene, on hybridization approaches designed to  
30 detect sequence changes or polymorphisms, or the use of antibodies to distinguish wild-type from

mutant/polymorphic hPXR. Changes that result in alteration of the DNA binding or ligand binding characteristics of hPXR can be expected to have a significant impact on hPXR activity. A mutation or  
5 polymorphism in hPXR can be indicative of a patient at increased risk of suffering an adverse reaction to a drug as a result of unusual rates of drug metabolism.

The invention also relates to antibodies,  
10 polyclonal or monoclonal, that are specific for hPXR, and antigen binding fragments thereof (e.g., Fab fragments). The antibodies can be generated in accordance with standard techniques using intact hPXR or a fragment thereof as defined above. The  
15 antibodies can be used, for example, in assays to detect the presence of the receptor. Further, the antibodies can be used in hPXR purification protocols.

The invention also relates to kits suitable for  
20 use, for example, in one or more method described above. The kits can include hPXR (or fragment thereof) or nucleic acid encoding same or antibodies as described above. The kit can also include compounds that bind hPXR, such as GW-485801. The  
25 hPXR, nucleic acid and/or antibody can be present in the kit disposed within a container means. The kit can also include ancillary reagents and buffers, etc., to facilitate practice of the specific method.

Certain aspects of the present invention are  
30 described in greater detail in the non-limiting Examples that follow.

### EXAMPLES

The following experimental details are relevant to the specific Examples that follow.

#### Chemicals

5       Dexamethasone-t-butylacetate and RU486 were purchased from Research Plus, Inc. (Bayonne, NJ) and Biomol (Plymouth Meeting, PA), respectively. All other compounds were purchased from either Sigma Chemical Co. (St. Louis, MO) or Steraloids, Inc.  
10       (Wilton, NH).

#### Molecular cloning of hPXR cDNAs

An EST was identified in the Incyte database (clone identification number 2211526) that contained nucleotides 444-2111 of the hPXR sequence. An  
15       oligonucleotide derived from this EST sequence (5' CTGCTGCGCATCCAGGACAT 3') (SEQ ID NO:1) was used to screen a pCMV-SPORT human liver cDNA library (Gibco/BRL) using Gene Trapper solution hybridization cloning technology (Gibco/BRL). Two  
20       clones were obtained that encoded hPXR, one containing nucleotides 1-2125, the other containing nucleotides 102-2118. The sequence of the longer is shown in Figure 1A. Sequences were aligned and analyzed by the University of Wisconsin Genetics  
25       Computer Group programs.

#### Plasmids

The expression vector pSG5-hPXR was generated by PCR amplification and subcloning of nucleotides

1-1608 of the hPXR clone into the pSG5 expression vector (Stratagene). pSG5-hPXR ATG was generated by PCR amplification of cDNA encoding amino acids 1-434 of hPXR using oligonucleotides 5'-

5 GGGTGTGGGGAATCCACCACCATGGAGGTGAGACCCAAAGAAAGC-3'  
(SEQ ID NO:2) (sense) and 5'-

GGGTGTGGGGGATCCTCAGCTACCTGTGATGCCG-3' (SEQ ID NO:3)  
(antisense) and insertion into EcoRI/BamHI-cut pSG5.

The bacterial expression vector pGEX-hPXR was  
10 generated by PCR amplification of cDNA encoding  
amino acids 108-434 and insertion into pGEX-2T  
(Pharmacia). The reporter plasmid (DR3)<sub>4</sub>-tk-CAT was  
generated by insertion of four copies of a double-  
stranded oligonucleotide containing the CYP3A1 DR3  
15 PXRE (5'-GATCAGACAGTTCATGAAGTTCATCTAGATC-3') (SEQ ID  
NO:4) into the BamHI site of pBLCAT2 (Luckow et al,  
Nucl. Acids Res. 15:5490 (1987)). The reporter  
plasmid (IR6)<sub>3</sub>-tk-CAT was generated by insertion of  
three copies of the CYP3A4 IR6 PXRE (5'-  
20 GATCAATATGAACTCAAAGGAGGTCAAGT-3') (SEQ ID NO:5) into  
the BamHI site of pBL2CAT. The pRSET-SRC1.14  
expression plasmid has been previously described  
(Kliwer, S.A., et al. Cell 92:73-82 (1998)). All  
constructs were confirmed by sequence analysis.

#### 25 Cotransfection assays

CV-1 cells were plated in 24-well plates in DME  
medium supplemented with 10% charcoal-stripped fetal  
calf serum at a density of  $1.2 \times 10^5$  cells per well.  
In general, transfection mixes contained 33 ng of  
30 receptor expression vector, 100 ng of reporter

plasmid, 200 ng of  $\beta$ -galactosidase expression vector (pCH110, Pharmacia), and 166 ng of carrier plasmid. Cells were transfected overnight by lipofection using Lipofectamine (Life Technologies, Inc.), according to the manufacturer's instructions. The medium was changed to DME medium supplemented with 10% delipidated calf serum (Sigma) and cells were incubated for an additional 24 hours. Cell extracts were prepared and assayed for CAT and  $\beta$ -galactosidase activities as previously described (Lehmann et al, J. Biol. Chem. 270:12953-12956 (1995)).

#### Northern analysis

An approximately 1.0 kb fragment encoding the LBD of hPXR was [ $^{32}$ P]-labeled by random priming and used to probe mouse multiple tissue Northern blots (Clontech). Blots were hybridized in ExpressHyb solution (Clontech) at 42°C overnight. Final washes were performed with 0.1x SSC, 0.1% SDS at 58°C.

#### Band shift assays

hPXR, mPXR1, and hRXR were synthesized *in vitro* using the TNT rabbit reticulocyte lysate coupled *in vitro* transcription/translation system (Promega) according to the manufacturer's instructions. Gel mobility shift assays (20  $\mu$ l) contained 10 mM Tris (pH 8.0), 40 mM KCl, 0.05% NP-40, 6% glycerol, 1 mM DTT, 0.2  $\mu$ g of poly(dI-dC) and 2.5  $\mu$ l each of *in vitro* synthesized PXR and RXR proteins. Competitor oligonucleotides were included



at a 10-fold or 50-fold excess. After a 10 min incubation on ice, 10 ng of [<sup>32</sup>P]-labeled oligonucleotide was added and the incubation continued for an additional 10 min. DNA-protein  
5 complexes were resolved on a 4% polyacrylamide gel in 0.5X TBE (1X TBE = 90 mM Tris, 90 mM boric acid, 2 mM EDTA). Gels were dried and subjected to autoradiography at -70°C. The following oligonucleotides were used as either radiolabeled  
10 probes or competitors (sense strand is shown):  
CYP3A4 IR6: 5' GATCAATATGAACTCAAAGGAGGTCAGTG 3'  
(SEQ ID NO:6)  
CYP3A4 IR6m1 5' GATCAATATGTTCTCAAAGGAGAACAGTG 3'  
(SEQ ID NO:7)  
15 CYP3A4 IR6m2 5' GATCAATAACAACCTCAAAGGAGGTCAGTG 3'  
(SEQ ID NO:8)  
CYP3A1 DR3: 5' GATGCAGACAGTTCATGAAGTTCATCTAGATC  
3' (SEQ ID NO:9).

#### CARLA

20 GST-hPXR fusion protein was expressed in BL21(DE3)plysS cells and bacterial extracts prepared by one cycle of freeze-thaw of the cells in Protein Lysis Buffer containing 10 mM Tris, pH 8.0, 50 mM KCl, 10 mM DTT, and 1% NP-40 followed by  
25 centrifugation at 40,000 x g for 30 minutes. Glycerol was added to the resulting supernatant to a final concentration of 10%. Lysates were stored at -80°C. [<sup>35</sup>S]SRC1.14 was generated using the TNT rabbit reticulocyte system (Promega) in the presence  
30 of Pro-Mix (Amersham). Coprecipitation reactions

included 25  $\mu$ l of lysate containing GST-hPXR fusion protein, 25  $\mu$ l Incubation Buffer (50 mM KCl, 40 mM HEPES pH 7.5, 5 mM  $\beta$ -mercaptoethanol, 1% Tween-20, 1% non-fat dry milk), 5  $\mu$ l [ $^{35}$ S]SRC1.14, and vehicle  
5 (1% DMSO) or compounds as indicated. The mixtures were incubated for 25 minutes at 4°C with gentle mixing prior to the addition of 15  $\mu$ l of glutathione-sepharose 4B beads (Pharmacia) that had been extensively washed with Protein Lysis Buffer.  
10 Reactions were incubated with gentle mixing at 4°C for an additional 25 min. The beads were pelleted at 3000 rpm in a microfuge and washed 3 times with Protein Incubation Buffer containing either vehicle alone, dexamethasone-t-butylacetate, rifampicin, or  
15 clotrimazole. After the last wash, the beads were resuspended in 25  $\mu$ l of 2X SDS-PAGE sample buffer containing 50 mM DTT. Samples were heated at 100°C for 5 minutes and loaded onto a 10% Bis-Tris PAGE gel. Gels were dried and subjected to  
20 autoradiography.

#### EXAMPLE 1

##### Molecular Cloning and Tissue Expression Pattern of hPXR

A human EST was identified in the Incyte  
25 LifeSeq® proprietary database that was highly homologous to a region of mPXR1 (Kliwer et al, Cell 92:73-82 (1998)). Two larger clones were isolated in a screen of a human liver cDNA library using an oligonucleotide within the EST as a probe. The

longest of these clones was 2146 bp in length (Fig. 1A) and encoded a new member of the nuclear receptor superfamily that was 97% and 76% identical to mPXR1 in the DNA binding domain (DBD) and ligand binding domain (LBD), respectively (Fig. 1B). In terms of other members of the nuclear receptor superfamily, hPXR was most closely related to the *Xenopus laevis* orphan receptor ONR1 (Smith et al, Nucl. Acids Res. 22:66-71 (1994)) and the vitamin D receptor (Fig. 1B). Notably, the hPXR sequence lacked an AUG initiator codon in between an in-frame stop codon (nucleotides 205-207 in the hPXR sequence) and the start of the region encoding the DBD. However, transfection experiments performed in CV-1 cells with the hPXR clone and a reporter plasmid containing four copies of an established mPXR binding site from the rat CYP3A1 gene promoter inserted upstream of the minimal thymidine kinase (tk) promoter and the chloramphenicol acetyltransferase (CAT) gene (Kliewer et al, Cell 92:73-82 (1998)) demonstrated that the hPXR clone encoded a functional nuclear receptor that was activated efficiently by dexamethasone-t-butylacetate, a known mPXR1 ligand (Kliewer et al, Cell 92:73-82 (1998)) (Fig. 1C).

Examination of the hPXR sequence revealed an in-frame CUG codon (nucleotides 304-306) surrounded by a favorable Kozak sequence (Kozak, J. Biol. Chem. 266:19867-19870 (1991)). There is precedent for the use of CUG codons to initiate translation of eukaryotic proteins, including the nuclear receptor

RAR $\beta$ 4 (Kozak, J. Biol. Chem. 266:19867-19870 (1991), Nagpal et al, Proc. Natl. Acad. Sci. USA 89:2718-2722 (1992)). Initiation of translation at this CUG codon would yield a protein of 434 amino acids, 5 three longer than mPXR1, with a predicted MW of 49.7 kD. In order to determine whether translation of the hPXR cDNA initiated at the CUG codon, hPXR RNA containing the wild-type 5' region was translated in the presence of [<sup>35</sup>S]methionine using rabbit 10 reticulocyte lysates. As a control, hPXR RNA, in which this CUG codon had been mutated to the optimal AUG (hPXR AUG), was also translated *in vitro*. Translation of the wild-type hPXR RNA resulted in an approximately 50 kD protein that co-migrated with 15 the translation product of hPXR AUG RNA (Fig. 1D, open arrow with asterisk). This 50 kD product was not produced when hPXR antisense RNA was used in the translation reaction. Much lower amounts of an approximately 53 kD translation product were also 20 produced in translation reactions performed with hPXR RNA (Fig. 1D, bent arrow), indicating that a small amount of translation initiated at other non-AUG codons upstream of the CUG codon. However, the results indicate that the CUG codon represents the 25 principal translation initiation site for hPXR containing a functional DBD.

The tissue expression pattern of hPXR was next examined via Northern analysis using blots containing poly(A)+ RNA prepared from multiple adult 30 tissues. hPXR mRNA was expressed most abundantly in

liver and was also present in the colon and small intestine (Fig. 2). Three transcripts of different size were detected in each of these tissues: a prominent 2.6 kb product and two less abundant messages of approximately 4.3 kb and 5 kb. It was recently shown that the mPXR gene is also abundantly expressed in liver and small intestine (Kliewer et al, Cell 92:73-82 (1998)). Whereas mPXR message was also detected at low levels in stomach and kidney, mRNA for hPXR was not detected in these tissues (Fig. 2). Thus, both hPXR and mPXR are most abundantly expressed in the liver and tissues of the gastrointestinal tract; however, there are differences in PXR expression patterns in mice and humans.

#### EXAMPLE 2

##### hPXR Activates Transcription Through a Response Element in the CYP3A4 Gene Promoter

Several lines of evidence have been provided that mPXR1 regulates CYP3A1 gene expression: mPXR1 was activated by compounds known to activate CYP3A1 gene expression including glucocorticoids and antiglucocorticoids, mPXR1 and CYP3A1 gene expression colocalized in the liver and small intestine, and mPXR1 bound to a response element in the CYP3A1 gene promoter that had previously been determined to confer responsiveness to glucocorticoids and antiglucocorticoids (Kliewer et al, Cell 92:73-82 (1998), Quattrochi et al, J. Biol.

Chem. 270:28917-28923 (1995), Huss et al, J. Biol. Chem. 93:4666-4670 (1996)). The findings that the CYP3A4 gene is also expressed in the liver and intestine and that this expression is induced in response to glucocorticoids and antiglucocorticoids (Molawa et al, Proc. Natl. Acad. Sci. USA 83:5311-5315 (1986), Kocarek et al, Drug Met. Dispos. 23:415-421 (1995)) led to the investigation of whether hPXR regulates CYP3A4 gene expression.

10       The induction of CYP3A4 expression in response to dexamethasone and rifampicin has been localized to an approximately 20 bp region of the promoter that contains two copies of the nuclear receptor half-site sequence AG(G/T)TCA organized as an inverted repeat (IR) and separated by 6 base pairs, an IR6 motif (Barwick et al, Molec. Pharmacol. 15 50:10-16 (1996)) (Fig. 3B). This IR6 motif is highly conserved in the promoters of CYP3A gene family members of several species (Barwick et al, Molec. Pharmacol. 50:10-16 (1996)). Interestingly, this half-site configuration is very different from that found in the CYP3A1 PXR response element (PXRE) which contains two half-sites organized as a direct repeat (DR) with a 3 nucleotide spacer, a DR3 motif (Kliewer et al, Cell 92:73-82 (1998)). To determine whether hPXR could regulate transcription through the IR6 motif, a reporter plasmid was generated containing three copies of the CYP3A4 IR6 response element upstream of the tk promoter and CAT gene.

20 25 30 Cotransfection assays were performed with the (IR6)<sub>3</sub>-tk-CAT reporter and pSG5-hPXR ATG expression

plasmids in CV-1 cells that were either treated with vehicle alone or 10  $\mu$ M dexamethasone-t-butylacetate. hPXR induced reporter levels in the presence of dexamethasone-t-butylacetate (Fig. 3A),

5 demonstrating that hPXR can activate transcription through the CYP3A4 IR6 motif.

In order to determine whether hPXR interacted directly with the CYP3A4 IR6 response element, band shift assays were performed. Since mPXR1 binds to  
10 DNA as a heterodimer with RXR (Kliewer et al, Cell 92:73-82 (1998)), it was suspected that hPXR would require RXR for high-affinity interactions with DNA. Neither hPXR nor RXR bound to a radiolabeled oligonucleotide containing the CYP3A4 IR6 motif on  
15 their own (Fig. 3C). However, hPXR and RXR bound efficiently as a heterodimer to the IR6 PXRE. The hPXR/RXR complex was competed efficiently by unlabeled oligonucleotides encoding either the IR6 PXRE from the CYP3A4 promoter or the DR3 PXRE from  
20 the CYP3A1 promoter that it was previously defined as a mPXR1/RXR binding site (Kliewer et al, Cell 92:73-82 (1998)) (Fig. 3C). Thus, the hPXR/RXR heterodimer interacted efficiently with two response elements with remarkably different architecture.  
25 Little or no competition was seen when competitor oligonucleotides were used that contained mutations in either the 5' half-site or both half-site sequences of the IR6 PXRE (Fig. 3C). The same binding profile was observed when the mPXR1 was  
30 substituted for hPXR (Fig. 3C). It was concluded from these experiments that hPXR binds efficiently

to the CYP3A4 IR6 PXRE as a heterodimer with RXR, and that hPXR and mPXR1 have very similar DNA binding profiles.

### EXAMPLE 3

#### 5 Differential Activation of Human and mPXR

CYP3A4 gene expression is induced in response to a remarkable array of xenobiotics, including synthetic steroids (Kocarek et al, Drug Met. Dispos. 23:415-421 (1995), Schuetz et al, J. Biol. Chem. 10 259:2007-2012 (1984), Heuman et al, Mol. Pharmacol. 21:753-760 (1982), Schulte-Hermann et al, Cancer Res. 48:2462-2468 (1988)), macrolide antibiotics (Wrighton et al, Biochem. 24:2171-2178 (1985)), antimycotics (Hostetler et al, Mol. Pharmacol. 15 35:279-285 (1989)), HMG-CoA reductase inhibitors (statins) (Kocarek et al, Toxicol. Appl. Pharmacol. 120:298-307 (1993), Schuetz et al, Hepatology 18:1254-1262 (1993)), and phenobarbital-like compounds (Heuman et al, Mol. Pharmacol. 21:753-760 20 (1982)). It was next determined whether hPXR might mediate the effects of some or all of these compounds on CYP3A4 expression. CV-1 cells were cotransfected with the pSG5-hPXR ATG expression plasmid and the (IR6)<sub>3</sub>-tk-CAT reporter plasmid, and 25 the cells were treated with micromolar concentrations of a number of compounds that are known to induce CYP3A gene expression in humans and/or rodents. As shown in Fig. 4A, hPXR was activated by the synthetic steroids dexamethasone,



dexamethasone-t-butylacetate, PCN, RU486, spironolactone, and cyproterone-acetate.

Dexamethasone-t-butylacetate and RU486 were the most efficacious activators of hPXR among the synthetic  
5 steroids tested. Notably, the antibiotic rifampicin and the antimycotic clotrimazole were both efficacious activators of hPXR (Fig. 4A). The antihypercholesterolemic drug lovastatin also activated hPXR as did phenobarbital and the  
10 organochlorine pesticide transnonachlor (Fig. 4A). Thus, hPXR is activated by a remarkably diverse group of synthetic compounds that are known to induce CYP3A4 gene expression (Fig. 4B).

Several naturally-occurring C21 steroids were  
15 also tested on hPXR that were previously shown to activate mPXR1 (Kliewer et al, Cell 92:73-82 (1998)). Pregnenolone, progesterone, and 5  $\beta$ -pregnane-3,20-dione all activated hPXR roughly 4-fold. The 17-hydroxy derivatives of pregnenolone  
20 and progesterone were weak activators of hPXR (Fig. 4A). These natural steroids all activated hPXR in transient transfection assays with EC<sub>50</sub> values >10  $\mu$ M, suggesting that they are unlikely to be natural hPXR ligands. However, related pregnanes or  
25 pregnane metabolites may serve as natural hPXR ligands.

Analyses of the effects of chemical inducers of CYP3A gene expression in primary hepatocytes obtained from either rodents or humans have revealed  
30 significant interspecies differences (Barwick et al,

Molec. Pharmacol. 50:10-16 (1996), Kocarek et al, Drug Met. Dispos. 23:415-421 (1995)). For example, rifampicin is an efficacious inducer of CYP3A4 gene expression in human hepatocytes but has little or no effect on CYP3A1 levels in rat hepatocytes. In contrast, PCN has marked effects on CYP3A levels in rat hepatocytes but only modest effects in human hepatocytes. To examine whether differences in PXR activation profiles might account for these interspecies variations, the same panel of compounds was tested on mPXR1. As shown in Fig. 4A, there were marked differences in the response profiles of the mouse and human homologs of PXR. Whereas rifampicin was an efficacious activator of hPXR, it was only a weak activator of mPXR1 (Fig. 4A). Clotrimazole, lovastatin and phenobarbital were also more efficacious activators of hPXR than mPXR1. In contrast, PCN only activated hPXR approximately 3-fold but activated mPXR1 roughly 9-fold (Fig. 4A). Taken together, these data indicate that much of the interspecies variability in CYP3A regulation may be due to differences in PXR activation profiles.

The panel of chemicals that induce CYP3A expression was also profiled on the human glucocorticoid receptor (GR). As shown in Fig. 4A, only dexamethasone and dexamethasone-t-butylacetate were efficacious activators of the GR. None of the other compounds activated the GR >1.5-fold (Fig. 4A). In contrast to a recent report (Calleja et al, Nature Med. 4:92-96 (1998)), activation of the GR by rifampicin was not observed. Since this previous

work was performed in HepG2 cells, it may be that rifampicin is differentially metabolized in various cell lines. As expected, neither pregnenolone, progesterone, nor their 17-hydroxy derivatives had an effect on GR activity (Fig. 4A). Thus, the broad activation profile that was observed for the human and mouse homologs of PXR with inducers of CYP3A gene expression is not a general property of other steroid hormone receptors.

10 In the absence of high-affinity radioligands, coactivator-based assays have been used as a biochemical means to determine whether compounds that activate orphan nuclear receptors do so through direct interactions with the protein (Kliwer et al, Cell 92:73-82 (1998), Krey et al, Mol. Endocrinol. 11:779-791 (1997)). These assays are predicated on the finding that ligands induce the interaction of nuclear receptors with accessory proteins, termed coactivators (Krey et al, Mol. Endocrinol. 11:779-791 (1997)). It was recently demonstrated that several steroidal activators of mPXR1, including dexamethasone-t-butylacetate and PCN, promote the interaction of the mPXR1 LBD with a 14 kD fragment of the steroid receptor coactivator 1 (SRC1.14) (Kliwer et al, Cell 92:73-82 (1998)). In order to examine whether the structurally-diverse compounds that activate hPXR do so by acting as ligands, three of the more potent activators representing different chemical classes were selected, dexamethasone-t-butylacetate, rifampicin, and clotrimazole, for testing in the coactivator-receptor ligand assay

(CARLA). The LBDs of hPXR and mPXR1 were expressed in *E. coli* as fusion proteins with glutathione-S-transferase (GST), and SRC1.14 was synthesized in vitro in the presence of [<sup>35</sup>S]methionine and  
5 [<sup>35</sup>S]cysteine. As shown in Fig. 4C, dexamethasone-t-butylacetate, rifampicin and clotrimazole each promoted the interaction of [<sup>35</sup>S]SRC1.14 with GST-hPXR. Consistent with the results of the transfection studies, dexamethasone-t-butylacetate  
10 induced an efficient interaction between GST-mPXR1 and [<sup>35</sup>S]SRC1.14 whereas rifampicin and clotrimazole did not (Fig. 4C). Taken together, these data indicate that structurally-divergent compounds can serve as hPXR ligands, and that the human and mouse  
15 homologs of PXR differ significantly in terms of their ligand binding properties.

#### EXAMPLE 4

##### Transfection Assay

Plasmids: GAL4-hPXR chimera and UAS-tk-SPAP  
20 reporters. The GAL4-hPXR expression constructs contain the translation initiation sequence and amino acids 1 to 147 of the yeast *S. crevisiae* transcription factor GAL4 in the pSG5 expression vector (Statagene). Amino acids 108 to 434 of hPXR  
25 are amplified by polymerase chain reaction (PCR) using vent polymerase (New England Biolabs) and inserted C-terminal to the GAL4 sequences. The UAS-tk-SPAP reporter contains 5 copies of the GAL4

binding site upstream of the tk promoter and the CAT gene (Berger et al, Gene 66:1 (1988)).

Transfection assay: SPAP reporter. CV-1 cells are plated in DME medium supplemented with 10% delipidated fetal calf serum at a density of  $2.4 \times 10^4$  cells per well in a 96-well plate (Costar) 16-24 h before transfection. In general, 8.0 ng of reporter plasmid, 25.0 ng of  $\beta$ -galactosidase expression vector (pCH110, Pharmacia), and 2.0 ng of GAL4-hPXR expression vector are mixed with carrier DNA (pBluescript, Stratagene) to a total of 80 ng of DNA per well in a volume of 10ml optiMEM I medium (Life Technologies). To this, a second mix, containing 9.3 ml optiMEM I medium and 0.7 ml of LIPOFECTAMINE<sup>TM</sup> (Life Technologies), is added. After 30 min., an additional 80ml of optiMEM I medium are added and the combined mix is then applied to the cells. Sixteen hours later, the medium is changed to DME medium supplemented with 10% delipidated and heat inactivated fetal calf serum and the test compound at a concentration of  $10^{-5}$ M. After incubation for 24 h, SPAP activity and  $\beta$ -galactosidase activity are measured by directly adding to the medium 200ml substrate mix (16mM o-nitrophenyl  $\beta$ -D-galactopyranoside (Sigma), 120mM fluorescein diphosphate (Molecular Probes), 0.16% Triton X-100, 160mM diethanolamine pH9, 44.8mM NaCl, and 0.8mM  $MgCl_2$ ). Alternatively, alkaline phosphatase and  $\beta$ -galactosidase activities are measured separately using standard protocols.

Briefly, cells are lysed by adding 25ml 0.5% Triton X-100 to the supernatant. To 40ml cell lysate, 200ml  $\beta$ -galactosidase substrate reagent (36mM o-nitrophenyl  $\beta$ -D-galactopyranoside, 1.25mM  $\text{MgCl}_2$ , 2.8mM NaCl, 4.4M  $\beta$ -mercaptoethanol) or 200ml alkaline phosphatase substrate reagent (2.5mM p-nitrophenyl phosphate, 0.5mM  $\text{MgCl}_2$ , 20mM NaCl, 1 M diethanolamine pH 9.85) are added and incubated for 1 h. Alkaline phosphatase activity is expressed as fold activation relative to that observed with vehicle alone (normalized to  $\beta$ -galactosidase activity which serves as internal control standard for transfection efficiency).

#### EXAMPLE 5

Synthesis of [ $^3\text{H}$ ]GW-485801

(i) The Preparation of [ $^3\text{H}$ ]3,5-Ditertbutyl-4-hydroxy benzaldehyde.

3,5-Ditertbutyl-4-hydroxy benzaldehyde, 5 mg (20.6  $\mu\text{mol}$ ) and Crabtree catalyst, 7.5 mg (9.3  $\mu\text{mol}$ ), were dissolved in 2 ml dichloromethane and stirred under 10 Ci tritium gas for 5 hours. The solution was then evaporated to dryness, and labile tritium was removed by repeated evaporations from methanol. The residue was redissolved in methanol, 10 ml, counted and analyzed.

Yield = 800 mCi.

Radiochemical purity by TLC on silica in hexane:ethyl acetate (80:20) was approximately 50%.

The crude material was evaporated to 1 ml and purified by preparative plate chromatography on a single 500  $\mu$ m silica plate, eluting in hexane:ethyl acetate (85:15). The plates were viewed under UV,  
5 the band corresponding to required aldehyde was collected and the product extracted into ethyl acetate. This was evaporated to dryness and redissolved in dichloromethane, counted and analyzed.

10 Yield = 370 mCi.

TLC as above showed a singly labelled, specific activity 23 Ci/mmol.

(ii) The Preparation of [ $^3$ H]GW-485801

The product from (i) above (370 mCi at 23  
15 Ci/mmol, 16  $\mu$ mol) was evaporated to dryness, redissolved in THF, 1 ml, and cooled in an ice bath with stirring. 1M Titanium (IV) chloride in toluene, 55  $\mu$ l, 55  $\mu$ mol, was added, immediate yellow color formed. Tetraethyl methylenediphosphonate, 75  
20  $\mu$ l, of a THF solution at 110 mg/ml, 28.6  $\mu$ mol, was added, followed by N-methyl morpholine, 8.1  $\mu$ l, 7.5 mg, 74  $\mu$ mol. This caused a deep blue color. The solution was then stirred at room temperature for 4 hours.

25 TLC analysis on silica in ethyl acetate:methanol (90:10) showed approximately 60% of the radioactivity to correspond to inactive GW-485801.

(iii) The Purification of [<sup>3</sup>H]GW-485801

The crude product was purified by preparative plate chromatography on 2 x 1mm silica plates, eluting in ethyl acetate:methanol (90:10). The plates were viewed under UV, the band corresponding to required product was collected and the product extracted into ethyl acetate:methanol (90:10). This was evaporated to dryness and redissolved in nitrogen-flushed ethanol, 30 ml. This was a yellow solution.

Yield = 180 mCi.

(iv) The Analysis of [<sup>3</sup>H]GW-485801

The purified product resulting from (iii) was analyzed by TLC, HPLC, mass spectroscopy and T-NMR.

TLC showed a radiochemical purity of 99%.

HPLC showed a radiochemical purity of 98.9%.

In both of the above systems, the radioactive peak co-eluted with inactive GW-485801.

Mass spectroscopy showed a specific activity of 23 Ci/mmol, the isotope distribution being 18.4% unlabelled, 81.6% <sup>1</sup> x <sup>3</sup>H. The spectrum of the radioactive material was consistent with that of the inactive GW-485801.

T-NMR showed a single labelling position (peak split into four signals by coupling to the phosphorus atoms) corresponding to labelling in the vinylic position of GW-485801. This corresponds to labelling in the aldehyde-H in the precursor.

A portion of the material was diluted to 1 mCi/ml with nitrogen-flushed ethanol and dispensed



as 1 X 2 mCi pack. The remainder was stored at  
~20°C (approximately 170 mCi).

# EXAMPLE 6

## Biotin-His6-PXR/RXRα Protein

5       The coding sequence representing amino acids  
130-434 of human PXR (Genbank AF061056) was  
subcloned into the pRSETa expression vector  
(Invitrogen). Sequence encoding a polyhistidine tag  
derived from an N-terminal PCR primer (MKKGHHHHHHG),  
10 (SEQ ID NO:10) was fused in-frame. The resulting  
encoded His6-PXR sequence was as follows:

MKKGHHHHHHGSERTGTQPLGVQGLTEEQRMIRELMDAQMKTFDTTFSHFK  
NFRLPGLVSSGCELPESLQAPSREEAAKWSQVRKDLCSLKVSLQLRGEDGSV  
15 WNYKPPADSGGKEIFSLLPHMADMSTYMFKGIISFAKVISYFRDLPIEDQIS  
LLKGAAFELCQLRFNTVFNAETGTWECGRLSYCLEDTAGGFQQLLLEPMLKF  
HYMLKKLQLHEEEYVLMQAIISLFSPDRPGVLQHRVVDQLQEQFAITLKSYIE  
CNRPQPAHRFLFLKIMAMLTIELRSINAQHTQRLRLRIQDIHPFATPLMQELFG  
ITGS (SEQ ID NO:11). ,

20

Restriction enzymes Nde I and Hind III were  
used to release the cDNA fragment encoding amino  
acids 225-462 of RXRα from BB5508 (pRSETa). The  
fragment was ligated into the like-cut pET24a  
25 expression plasmid (Novagen). The Bgl II, Hind III  
fragment (contains T7 promoter, lac operator, RBS  
and RXRα) of this construct was then cloned into the  
BamH I, Hind III sites (removes tetracycline  
resistance) of pACYC184 (BB5114). This allows for

expression of RXR $\alpha$  from the T7 promoter when grown in BL21(DE3) cells and induced with IPTG. The resulting encoded RXR $\alpha$  sequence was as follows:

5 MKKGSANEDMPVERILEAELAVEPKTETYVEANMGLNPSSPNPVTNICQAA  
DKQLFTLVWAKRIPHFSELPLDDQVILLRAGWNELLIASFSHRSLAVKDGI  
LLATGLHVHRNSAHSAGVGAI FDRVLTELVSKMRDMQMDKTELGC LRAIVLF  
NPDSKGLSNPAEVEALREKVYASLEAYCKHKYPEQGRFAKLLLRPALRSI  
GLKCLEHLFFFKLIGDTPIDTFLMEMLEAPHQMT (SEQ ID NO:12).

10

The His6-PXR/pRSETa and RXR $\alpha$ /pACYC184 plasmids were cotransformed into the BL21(DE3) *E. coli* strain. One-liter shake flask liquid cultures containing standard Luria-Bertani (LB) broth with  
15 0.05 mg/ml Ampicillin and 0.05 mg/ml Chloramphenicol were inoculated and grown at 22°C for 24 hours. The cells were induced with 0.05 mM IPTG for 4-6 hours at 22°C then the cells were harvested by centrifugation (20 minutes, 3500 g, 4°C). The cell  
20 pellet was stored at -80°C. The cell pellet was resuspended in 250 ml Buffer A (50 mM Tris-Cl pH8.0, 250 mM NaCl, 50 mM imidazole pH7.5). Cells were sonicated for 3-5 minutes on ice and the cell debris was removed by centrifugation (45 minutes, 20,000g,  
25 4°C). The cleared supernatant was filtered through a 0.45  $\mu$ m filter and loaded on to a 50 ml ProBond [Ni<sup>++</sup> charged] chelation resin (Invitrogen). After washing to baseline with Buffer A, the column was washed with Buffer A containing 125 mM imidazole pH  
30 7.5. The His6-PXR/RXR $\alpha$  complex was eluted from the

column using Buffer A with 300 mM imidazole pH 7.5. Column fractions were pooled and concentrated using Centri-prep 30K (Amicon) units. The protein was subjected to size exclusion, using a column (26 mm X  
5 90 cm) packed with Sepharose S-75 resin (Pharmacia) pre-equilibrated with 20mM Tris-Cl pH 8.0, 200 mM NaCl, 5 mM DTT, 2.5 mM EDTA, pH 8.0. Column fractions were pooled and concentrated as before. The purified His6-PXR/RXR $\alpha$  was buffer exchanged by  
10 gel filtration into PBS, resulting in an average total molar protein concentration of 45mM. A five-fold total molar excess of NHS-LC-Biotin (Pierce) was added to this protein mixture in a minimal volume of PBS. This solution was incubated with  
15 gentle mixing for 60 minutes at ambient temperature, approximately 23°C. The biotinylation modification reaction was stopped by the addition of a 2000x molar excess of Tris-HCl, pH 8. The biotin-His6-PXR/RXR $\alpha$  was dialyzed at 4°C against 3 buffer  
20 changes, each of at least 50 volumes, TBS pH 8 containing 5mM DTT, 2mM EDTA and 2% sucrose. The biotin-His6-PXR/RXR $\alpha$  was subjected to mass spectrometric analysis to reveal the extent of modification by the reagent. The biotinylated  
25 protein solution was frozen and stored at -80°C.

EXAMPLE 7

## PXR Scintillation Proximity Assay (SPA)

Streptavidin-PVT SPA beads (Amersham Pharmacia cat # RPNQ0007) were resuspended in assay buffer (50 mM Tris HCl pH 8.0, 50 mM KCl, 1 mM DTT, 0.1 mg/ml essentially fatty acid free bovine serum albumin) at 0.5 mg/ml. Biotin-His6-PXR/RXR $\alpha$  was added to the beads to a final concentration of 50 nM. The receptors were allowed to couple to the SPA beads for thirty minutes at room temperature. The uncoupled receptor was removed by centrifuging the SPA beads at 3000rpm for 5 minutes in a swinging bucket rotor of a Rupp & Bowman Silencer centrifuge. The receptor coated SPA beads were then resuspended in assay buffer to 3.3 mg/ml. 100  $\mu$ g (30  $\mu$ L) of receptor coated SPA beads were added to each well of a 96-well Optiplate (Packard cat # 6005190). Each well also contained [ $^3$ H]GW-485801 at final concentrations ranging from 0.5 nM to 800 nM. Non-specific binding was determined by addition of 10  $\mu$ M clotrimazole. The total volume in each well was 100  $\mu$ L. The plates were sealed with TopSealA (Packard cat # 6005185) and agitated momentarily to ensure complete mixing. The plates were then allowed to incubate at room temperature until equilibrium was obtained. The plates were then counted on a TopCount liquid scintillation counter (Packard) using a protocol optimized for  $^3$ H PVT SPA. Triplicate samples in the absence (T samples) or

presence (NS samples) of clotrimazole were averaged and specific binding was calculated using the equation:

5                      specific binding = T-NS

Plots of specific binding vs concentration of [<sup>3</sup>H]GW-485801 were generated (Fig. 6). K<sub>d</sub> values were determined using non-linear regression when the data  
10 were fit to the equation of a rectangular hyperbola.

Test compounds were dissolved in DMSO at 10 mM and diluted 1:10 in DMSO before serially diluting in assay buffer. Compounds were typically tested at concentrations ranging from 100 μM to 0.3 nM.

15 Streptavidin-PVT SPA beads (Amersham Pharmacia cat # RPNQ0007) were resuspended in assay buffer (50 mM Tris HCl pH 8.0, 50 mM KCl, 1 mM DTT, 0.1 mg/ml essentially fatty acid free bovine serum albumin) at 0.5 mg/ml. Biotin-His6-PXR/RXRα was added to the  
20 beads to a final concentration of 50 nM. The receptors were allowed to couple to the SPA beads for thirty minutes at room temperature. The uncoupled receptor was removed by centrifuging the SPA beads at 3000 rpm for 5 minutes in a swinging  
25 bucket rotor of a Rupp & Bowman Silencer centrifuge. The receptor coated SPA beads were then resuspended in assay buffer to 3.3 mg/ml. 100 μg (30 μL) of receptor coated SPA beads was added to each well of a 96-well Optiplate (Packard cat # 6005190). Each  
30 well also contained [<sup>3</sup>H]GW-485801 at a final

concentration of 25 nM and test compound or an equal volume of assay buffer. Non-specific binding was determined by addition of 10  $\mu$ M clotrimazole. The total volume in each well was 100  $\mu$ L. The plates were sealed with TopSealA (Packard cat # 6005185) and agitated momentarily to ensure complete mixing. The plates were then allowed to incubate at room temperature until equilibrium was obtained, approximately 1.5 hours. The plates were then counted on a TopCount liquid scintillation counter (Packard) using a protocol optimized for  $^3\text{H}$  PVT SPA and programmed to correct for color quenching. Values for "% [ $^3\text{H}$ ]GW-485801 Bound" were calculated using the following equation:

$$\% \text{ } [^3\text{H}]\text{GW-485801 Bound} = 100 * [(C_{\text{DPM}} - \text{NS}_{\text{DPM}}) / (T_{\text{DPM}} - \text{NS}_{\text{DPM}})]$$

where  $C_{\text{DPM}}$  is the DPM value from a well containing a test compound,  $\text{NS}_{\text{DPM}}$  is the average of the DPM values from the "non-specific" wells which contained 10  $\mu$ M clotrimazole,  $T_{\text{DPM}}$  is the average of the DPM values from the "total" wells which contained no added compounds. Graphs of "% [ $^3\text{H}$ ]GW-485801 Bound vs concentration were generated for each test compound and  $\text{IC}_{50}$  values were determined using non-linear regression (see Table 1).

Table 1

Compound	IC50 ( $\mu$ M)
GW-485801	0.58
Clotrimazole	1.3
Rifampicin	2.4
5b-pregnane-3,20-dione	1.0

\* \* \*

All documents cited above are hereby incorporated in their entirety by reference.

- 5 One skilled in the art will appreciate from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention.

WHAT IS CLAIMED IS:

1. A method of treating cholestatic liver disease comprising administering to a patient in need of such treatment an amount of a compound that induces CYP3A4 gene expression sufficient to effect said treatment, wherein said compound is identifiable using a method comprising the steps of:

i) contacting a test compound with the ligand binding domain of hPXR,

ii) determining whether said test compound binds to said ligand binding domain, wherein binding of the test compound to said ligand binding domain is indicative of a compound that induces CYP3A4 gene expression.



1/8

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 61 GACGGGAAGAGGAAGCACTGCCTTTACTTTCAGTGGGAATCTCGGCCTCAGCCTGCAAGCC  
 121 AAGTGTTCACAGTGAGAAAAGCAAGAGAATAAGCTAATACTCCTGTCTGAAACAAGGCAG  
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 241 CCCAGGGGAGAAGTCGGAGCAAGAAGCTTACCACCAAGCAGTCCAAGAGGCCCAAGCA  
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 L E V R P K E S W N H A D F V H C E D 19  
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 1861 AGGACCATCAGAGAGGCAAGGTTGCCCTTTCTTTTAAAGGCCCTGTGGTCTGGGGAGA  
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Figure 1A

2/8

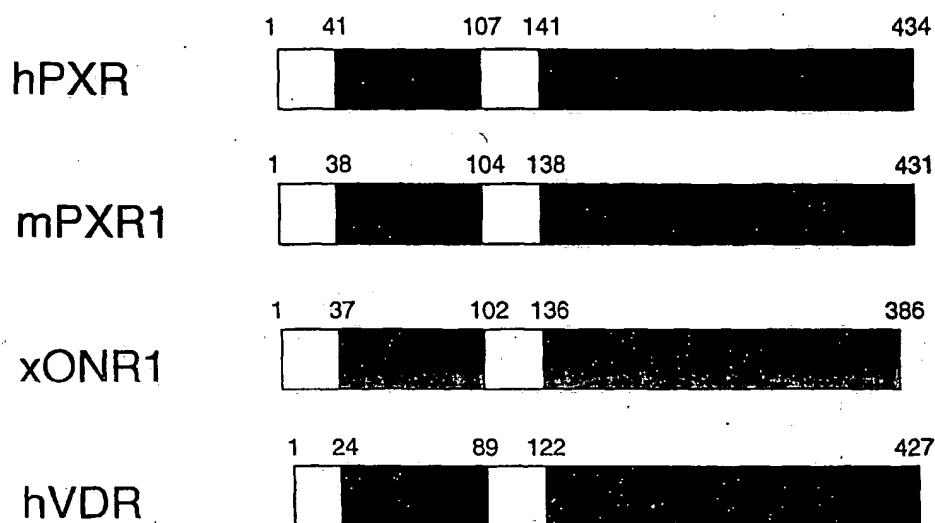


Figure 1B

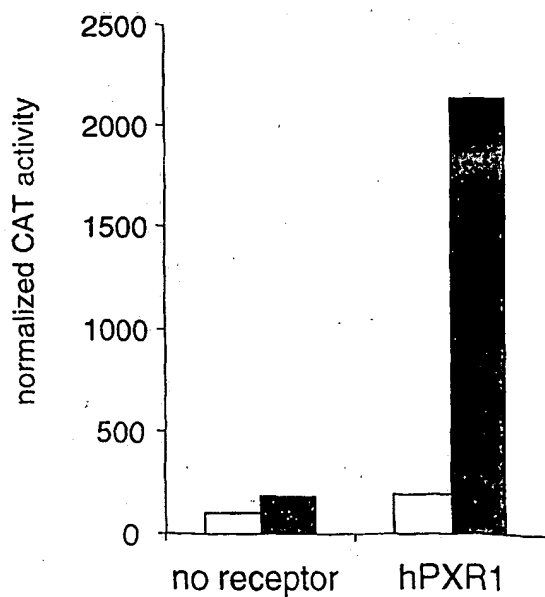


Figure 1C

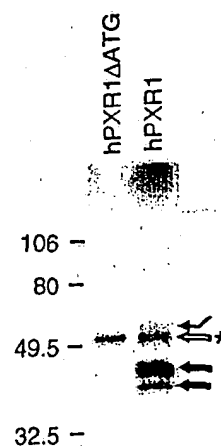


Figure 1D

3/8

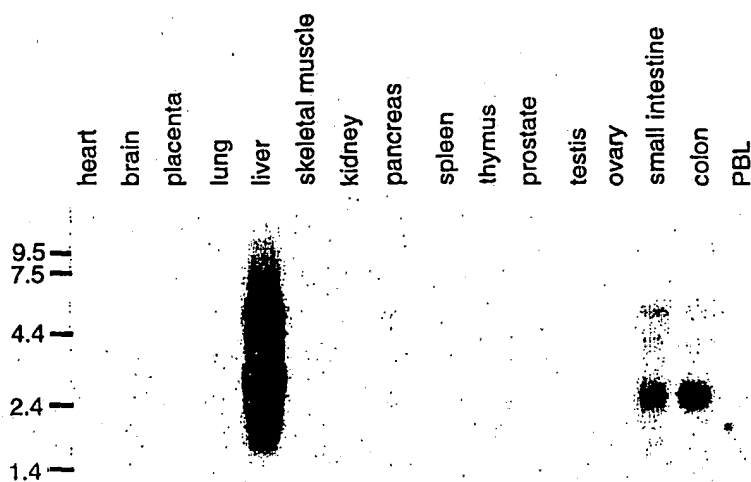


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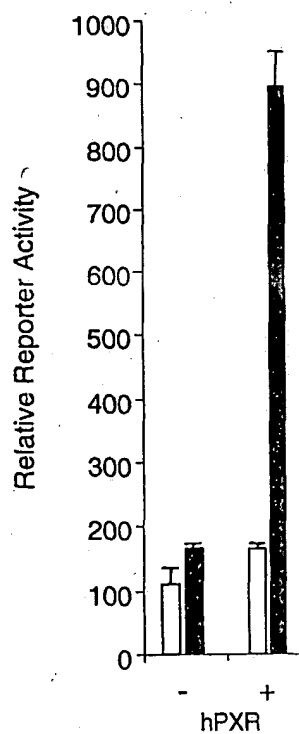


Figure 3A

4/8

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		<-xx--		--xx->	
CYP3A4 IR6 m2	ata	<b>ACAACT</b>	caaagg	<b>AGGTCA</b>	gtg
		xx----		----->	
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Figure 3B

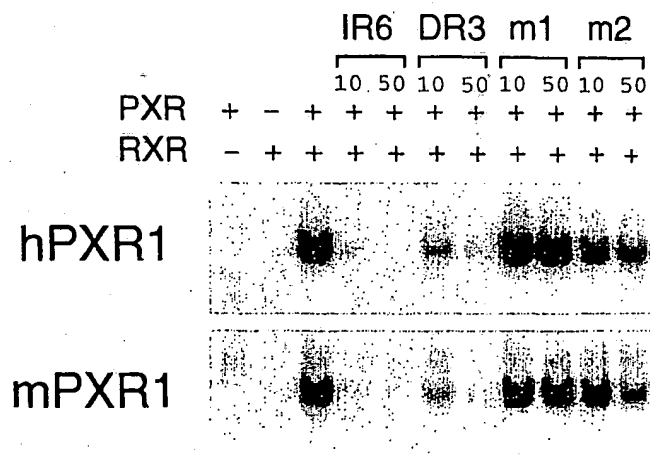


Figure 3C

5/8

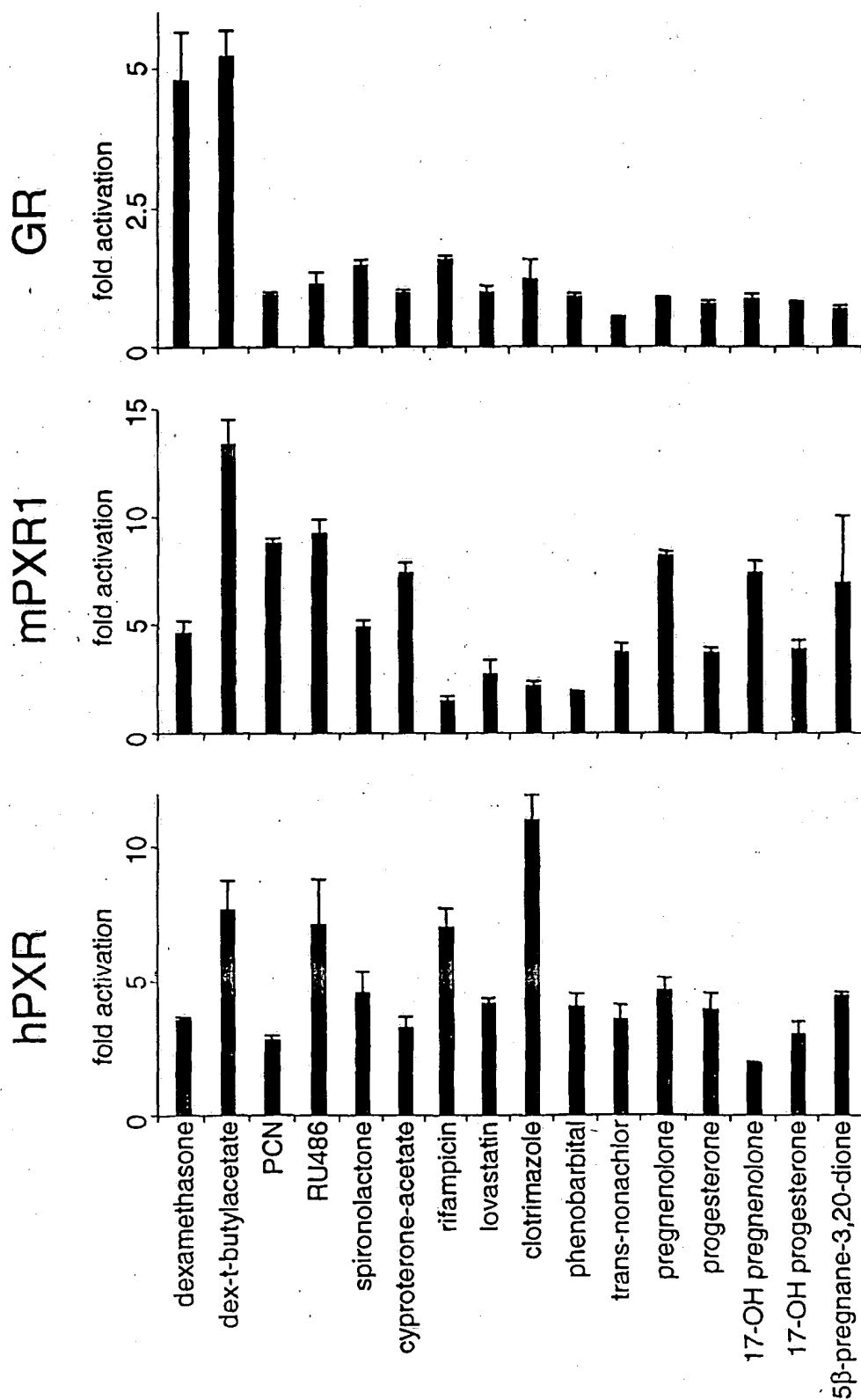


Figure 4A

6/8

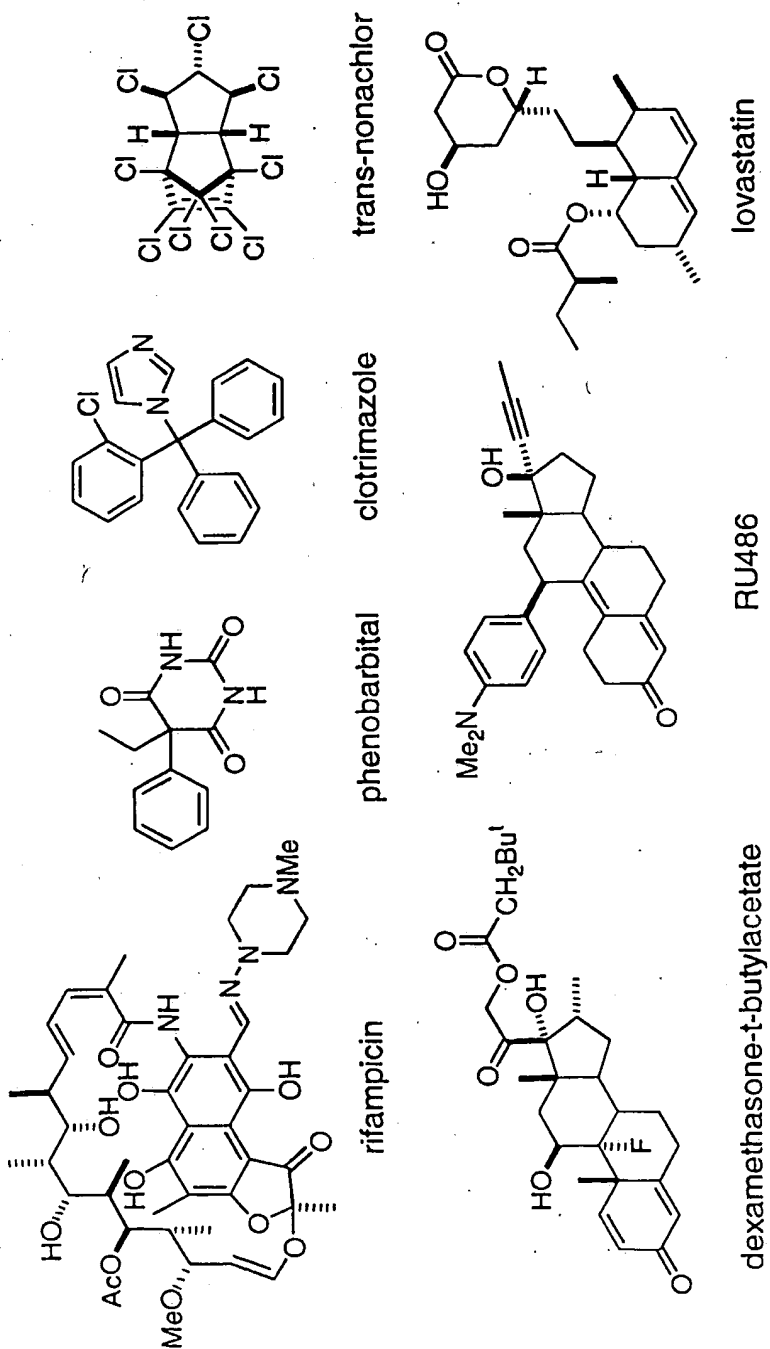


Figure 4B

7/8

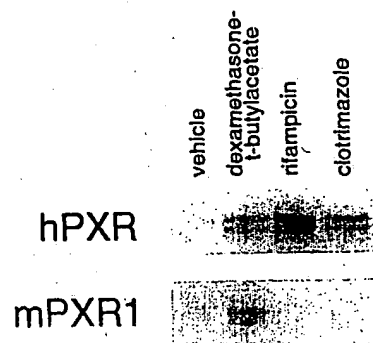


Figure 4C

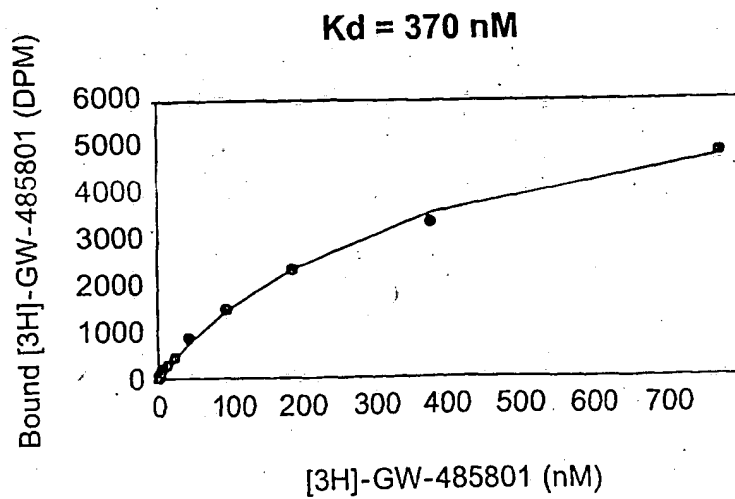


Figure 6

8/8

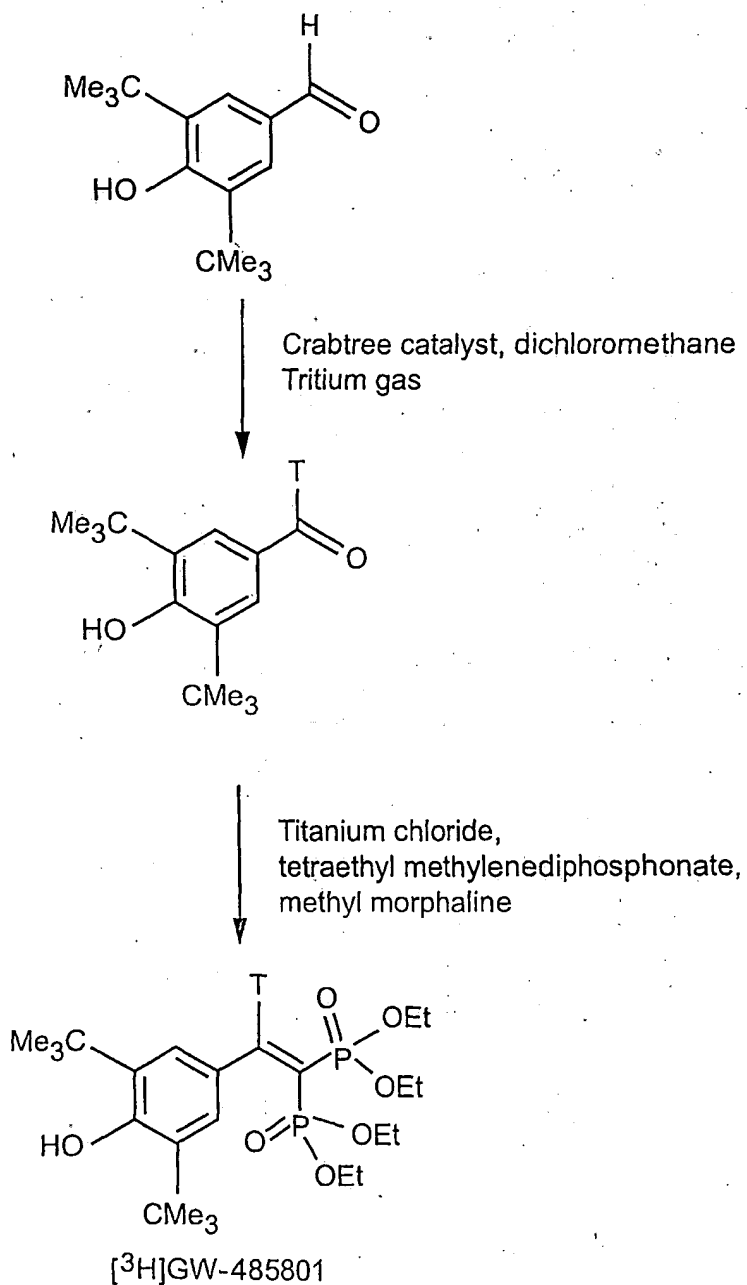
The Preparation of [<sup>3</sup>H]GW-4858011. REACTION SCHEME

Figure 5



## SEQUENCE LISTING

<110> KLEWER, Steven A.  
 JONES, Stacey A.  
 WILLSON, Timothy M.

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Ser His Phe Lys Asn Phe Arg Leu Pro Gly Val Leu Ser Ser Gly Cys  
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Glu Leu Pro Glu Ser Leu Gln Ala Pro Ser Arg Glu Glu Ala Ala Lys  
 65 70 75 80

Trp Ser Gln Val Arg Lys Asp Leu Cys Ser Leu Lys Val Ser Leu Gln  
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Leu Arg Gly Glu Asp Gly Ser Val Trp Asn Tyr Lys Pro Pro Ala Asp  
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Tyr Phe Arg Asp Leu Pro Ile Glu Asp Gln Ile Ser Leu Leu Lys Gly  
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Ala Ala Phe Glu Leu Cys Gln Leu Arg Phe Asn Thr Val Phe Asn Ala  
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Arg Ile Pro His Phe Ser Glu Leu Pro Leu Asp Asp Gln Val Ile Leu  
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His Arg Asn Ser Ala His Ser Ala Gly Val Gly Ala Ile Phe Asp Arg  
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 aggaccatca gagaggcaag gttgcccttt ccttttaaaa ggccctgtgg tctggggaga 1920  
 aatccctcag atccactaa agtgtcaagg tgtggaaggg accaagcgac caaggatagg 1980  
 ceatctgggg tctatgcca cataccacag ttgttctgct tcctgagctt ttctcattgct 2040  
 acctctaata gtctgtctc ccacttccca ctggttcccc tcctcttccg agctgctttg 2100  
 tgggctccag gcctgtactc atcgccaggt gcatgagtat ctgtgg 2146

&lt;210&gt; 14

&lt;211&gt; 414

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: Protein

&lt;400&gt; 14

Leu Glu Val Arg Pro Lys Glu Ser Trp Asn His Ala Asp Phe Val His  
 1 5 10 15

Cys Glu Asp Thr Glu Ser Val Pro Gly Lys Pro Ser Val Asn Ala Asp  
 20 25 30

Glu Glu Val Gly Gly Pro Gln Ile Cys Arg Val Cys Gly Asp Lys Ala  
 35 40 45

Thr Gly Tyr His Phe Asn Val Met Thr Cys Glu Gly Cys Lys Gly Phe  
 50 55 60

Phe Arg Arg Ala Met Lys Arg Asn Ala Arg Leu Arg Cys Pro Phe Arg  
 65 70 75 80

Lys Gly Ala Cys Glu Ile Thr Arg Lys Thr Arg Arg Gln Cys Gln Ala  
 85 90 95

Cys Arg Leu Arg Lys Cys Leu Glu Ser Gly Met Lys Lys Glu Met Ile  
 100 105 110

Met Ser Asp Glu Ala Val Glu Glu Arg Arg Ala Leu Ile Lys Arg Lys  
 115 120 125

Lys Ser Glu Arg Thr Gly Thr Gln Pro Leu Gly Val Gln Gly Leu Thr  
 130 135 140

Glu Glu Gln Arg Met Met Ile Arg Glu Leu Met Asp Ala Gln Met Lys  
 145 150 155 160

Thr Phe Asp Thr Thr Phe Ser His Phe Lys Asn Phe Arg Leu Pro Gly  
 165 170 175

Val Leu Ser Ser Gly Cys Glu Leu Pro Glu Ser Leu Gln Ala Pro Ser  
 180 185 190

Arg Glu Glu Ala Ala Lys Trp Ser Gln Val Arg Lys Asp Leu Cys Ser  
 195 200 205

Leu Lys Val Ser Leu Gln Leu Arg Gly Glu Asp Gly Ser Val Trp Asn  
 210 215 220

Tyr Lys Pro Pro Ala Asp Ser Gly Gly Lys Glu Ile Phe Ser Leu Leu  
 225 230 235 240

Pro His Met Ala Asp Met Ser Thr Tyr Met Phe Lys Gly Ile Ile Ser  
 245 250 255

Phe Ala Lys Val Ile Ser Tyr Phe Arg Asp Leu Pro Ile Glu Asp Gln  
 260 265 270

Ile Ser Leu Leu Lys Gly Ala Ala Phe Glu Leu Cys Gln Leu Arg Phe  
 275 280 285  
 Asn Thr Val Phe Asn Ala Glu Thr Gly Thr Trp Glu Cys Gly Arg Leu  
 290 295 300  
 Ser Tyr Cys Leu Glu Asp Thr Ala Gly Gly Phe Gln Gln Leu Leu Leu  
 305 310 315 320  
 Glu Pro Met Leu Lys Phe His Tyr Met Leu Lys Lys Leu Gln Leu His  
 325 330 335  
 Glu Glu Glu Tyr Val Leu Met Gln Ala Ile Ser Leu Phe Ser Pro Asp  
 340 345 350  
 Arg Pro Gly Val Leu Gln His Arg Val Val Asp Gln Leu Gln Glu Gln  
 355 360 365  
 Phe Ala Ile Thr Leu Lys Ser Tyr Ile Glu Cys Asn Arg Pro Gln Pro  
 370 375 380  
 Ala His Arg Phe Leu Phe Leu Lys Ile Met Ala Met Leu Thr Glu Phe  
 385 390 395 400  
 Ala Thr Pro Leu Met Gln Glu Leu Phe Gly Ile Thr Gly Ser  
 405 410